DAIDS

VIROLOGY MANUAL

FOR HIV LABORATORIES

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Compiled by

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NATIONAL INSTITUTES OF HEALTH

and

COLLABORATING INVESTIGATORS

QUALITATIVE PBMC MACROCOCULTURE ASSAY

I. PRINCIPLE

A co-culture of peripheral blood mononuclear cells (PBMC) and uninfected PHA-stimulated PBMC are maintained under ideal conditions to allow viral reproduction in vitro. Virtually all cell cultures (>95%) from HIV-1 seropositive patients will yield detectable HIV-1 antigen by this method.

II. SPECIMEN REQUIREMENTS

The assay utilizes ACD, CPD or heparin anticoagulated peripheral blood (minimum volume of 10 to 20 mL from adults or children, 1 to 2 mL from infants). The blood must be kept at room temperature until processing and should be processed within 30 hours.

III. REAGENTS

All reagents are prepared using deionized water, reagent grade I.

Sterile Phosphate Buffered Saline (PBS) or sterile Hank's Balanced Salt Solution (HBSS) without calcium or magnesium. Store at room temperature. Note manufacturer's outdate or discard one week after opening.

Sterile Ficoll-Hypaque or Lymphocyte Separation Medium (LSM) - Store at room temperature in the dark. Note manufacturer's outdate and date opened.

Penicillin - available in $5x10^6$ unit vials. Store at room temperature. Observe manufacturer's outdate.

a. Add 25 mL of sterile water to the vial. Mix until contents are dissolved. Final concentration = 200,000 units/mL

b. Divide into 0.32 mL aliquots in sterile 1.5 mL microcentrifuge tubes and freeze at -20^0C in a labeled box. Label with a 1 year outdate.

Gentamicin - available in 50 mg/mL bottles. Open bottles under laminar flow hood only; divide into 0.640 mL aliquots in sterile 1.5 mL microcentrifuge tubes. Store unopened bottles at room temperature; store aliquots at 4^0C. Outdates one month after opening.

Fetal Bovine Serum (FBS) - available in 500 mL sterile bottles from various manufacturers. Store frozen at -20^0C. Note manufacturer's outdate. When needed, rapidly thaw a bottle in a 37^0C water bath, then heat-inactivate in a 56^0C water bath for 30 minutes with occasional shaking. The

level of H_2O in the water bath should be as high as the level of the serum in the bottle. Store at 4^0C after thawing. Heat-inactivated FBS has a one month outdate.

RPMI 1640 medium with L-glutamine (2 mM) - Store at 4^0C and observe manufacturer's outdate.

IL-2 (interleukin-2) - available in 50 mL bottles from Boehringer Mannheim at a discounted price for labs within the ACTG. Store at -20^0C. Note manufacturer's outdate. As needed, thaw a 50 mL bottle (freeze the remaining 25 mL).

Basic Medium:

To make 620 mL:

a. Add 120 mL FBS to 500 mL of RPMI 1640 medium with L-glutamine. Final concentration (120/620) is approximately 20%.

b. Add 310 µL stock penicillin. (Concentration of penicillin used is $5x10^6$ units/25 mL or 200,000 units/mL; 0.31 mL of 200,000 units/mL = 62,000 units and 62,000 units/620 mL final volume of medium = 100 units/mL for final concentration).

c. Add 620 µL Gentamicin. (Concentration of Gentamicin used is 50 mg/mL or 50 µg/µL; 620 µL of 50 µg/µL = 31,000 µg and 31,000 µg/620 mL final volume of medium = 50 µg/mL for final concentration).

Store Basic Medium at 4^0C for up to 1 month.

Growth Medium - also called IL-2 Medium or T-Cell Growth Factor (TCGF) Medium.

To make 500 mL:

a. 475 mL Basic Medium.

b. 25 mL IL-2. (Final concentration = 25 mL/500 mL = 5%.)

Store Growth Medium at 4^0C for up to 1 month. Growth Medium should warmed before use.

Trypan Blue Stain - this stains non-viable cells dark blue, and is used to determine the viable cell count of a culture. Prepare a 0.4% solution by adding 0.4 gm Trypan Blue (available from Sigma) and 1 mL Glacial Acetic Acid to 99 mLs distilled H_20 or saline. After dissolving, filter solution through Whatman filter paper or a 0.45 µ filter.

PHA-stimulated uninfected donor PBMCs - see procedure for Preparation of PHA-stimulated, Uninfected Donor Peripheral Blood Mononuclear Cells (PBMC).

IV. EQUIPMENT AND SUPPLIES

Gloves
Disposable lab coat
Laminar flow hood (Class 2 biosafety hood).
Accuspin tubes with Ficoll, available from Sigma in 12 mL or 50 mL size tubes.
Sterile 15 and 50 mL conical tubes.
Sterile 2, 5, 10, and 25 mL pipettes.
Hemacytometer
Sterile 25 cm^2 tissue culture flask.
Sterile 500 mL bottles.
Sterile 1.5 and 0.5 mL microcentrifuge tubes.
20 µL, 200 µL, and 1000 µL pipetteman.
Sterile 200 µL and 1000 µL pipette tips.
Bleach (household bleach diluted 1/100 with tap water).
Centrifuge capable of speeds up to 1500 x g and equipped with a horizontal rotor and O- ring sealed safety cups.
Compound microscope.
CO_2 incubator (37 ± 1^0C with humidity).
37^0C and 56^0C water baths.
Pipette aid.

V. PROCEDURE

1. Log patient information into the lab computer and label specimen with the assigned specimen number.

2. Obtain PBMC as follows. Carefully label all tubes and flasks for each sample of blood being processed.

NOTE: SUBSEQUENT PROCEDURES SHOULD BE PERFORMED IN A CLASS 2 BIOSAFETY LAMINAR FLOW HOOD USING STERILE TECHNIQUE AND ADHERING TO CDC/NIH STANDARDS (INCLUDING USE OF GLOVES AND LAB COATS).

3. Separate PBMC from the blood as follows:

    a. Accuspin Method: Carefully pour blood into Accuspin tubes. If plasma is being removed for storage, rinse original blood tubes with a volume of PBS or HBSS equal to the volume of plasma removed and add to the Accuspin

tubes. (Use 1-2 small Accuspin tubes for blood volumes less than 15 mL.) Centrifuge the tubes at room temperature at 800 x g for 20 minutes.

b. Overlay Method: If plasma is being removed for storage, rinse original blood tubes with a volume of PBS of HBSS equal to the volume of plasma removed and add back to cells and remaining plasma. If whole blood is being used, add one part PBS of HBSS to one part whole blood. Blood should be carefully overlaid at a ratio of 4 parts diluted blood to 3 parts Ficoll reagent in a 15 or 50 mL sterile tube depending on the volume of blood to be separated. Centrifuge tubes at room temperature at 400 x g for 30 minutes.

c. CPT tubes: The CPT tubes should be centrifuged at 1500 x g for 20 mins. in a horizontal rotor within 3 hrs. of collection. If the tubes are drawn off site and must be mailed, they should be centrifuged and then resuspended before shipping. They can be recentrifuged as above. NOTE: CPT tubes are made of glass and can fracture during the high speed centrifugation required for the procedure. Care should be taken when removing tubes from safety cups after spinning to avoid possible injury.

4. After centrifugation, remove cloudy interface (PBMC layer) into appropriately labeled 50 mL centrifuge tubes.

5. Wash cells by filling tube with sterile PBS or HBSS and centrifuge at 400 x g for 10 minutes.

6. Decant supernatant after centrifugation, resuspend cells and fill tube with sterile PBS or HBSS and wash again.

7. Resuspend the pellet in 10 mL of Growth Medium.

8. Count and record the number of viable PBMC/mL. Also record the ID number of the Donor PBMC to be used.

a. Pipette 10 µL of the sample into a 0.5 mL microcentrifuge tube, add 90 µL of Trypan blue stain and mix.

b. Load a hemacytometer and count the number of PBMC in the four large cells.

c. Calculate the number of PBMC/mL: $\frac{\text{PBMC in all four squares}}{4} \times 10^5$

Example: $\frac{88}{4} \times 10^5 = 2.2 \times 10^6$ PBMC/mL

Automated counting could be used, following manufacturer's procedures.

9. Set up culture as follows:

   Place a volume of patient cell suspension equal to $10x10^6$ PBMC in a labeled 25 cm^2 flask, add $10x10^6$ PHA-stimulated donor PBMC and bring the volume up to a total of 10 mL with Growth Medium. NOTE: If there are not enough patient cells, a culture may be set up using equal number of patient PBMC and donor cells at $2x10^6$ cells /mL, in a minimum volume of 5 mL per flask. Also, see Quality Control section. For very low patient cell number see Qualitative PBMC Micrococulture Procedure.

10. Incubate at 37^0C, 5% CO_2 with humidity.

11. Store remaining patient PBMC according to each protocol (viable PBMC, pellets, etc.). (See "Specimen Codes," "Specimen Processing" and "Specimen Storage Recommendations").

12. Feed cultures and harvest samples for HIV p24 antigen testing as follows:

    a. On day 3 or 4, remove 5 mL of supernatant from the flask without disturbing the cells (freeze a 1 mL aliquot for HIV p24 antigen detection) and replace with 5 mL of Growth Media. If less than 10 mL were used for the culture, remove one half the volume and replace with an equal volume of fresh medium.

    b. On day 7, remove 5 mL of supernatant and again freeze a 1 mL aliquot for testing. Replace with 5 mL Growth Media, including $10x10^6$ PHA-stimulated donor PBMC.

    c. Continue sampling and feeding in this manner until the end of culture: sampling twice per week, fresh media once per week and fresh media containing donor PBMC once per week.

13. A computer-generated sampling list is made every feeding day to identify cultures that need an aliquot of supernatant saved for testing. Save aliquots in tubes and store at -20^0C until tested for p24 antigen.

14. Maintain cultures for 21 days or until culture meets criteria for positivity.

15. At the end of culture:

    a. If the culture is negative, check an aliquot of cells for viability in order to detect a false negative due to cytotoxicity.

b. If the culture is positive, store samples according to each protocol (viable cultured PBMC, supernatant, etc.). (Please see "Specimen Processing" and "Specimen Codes.")

VI. QUALITY CONTROL

It is important to distinguish whether the priority for culturing the virus is to obtain an isolate or to make a diagnosis of HIV infection. If the latter, the standardization of patient and donor cell number and volume of media in flask is important. A positive culture is interpretable no matter how many cells were used for culture, but a negative culture may not be reliable for diagnosis if too few cells were used. A comment should be made in the computer if a nonstandard culture was set up.

VII. REPORTING RESULTS

Qualitative cultures whose supernatant p24 antigen results remain below the cutoff until at least day 21 are considered culture negative.

Qualitative cultures whose supernatant meet one of the following criteria are considered culture positive:

Two consecutive HIV p24 antigen VQA **corrected** values of > 30 pg/mL, of which the second value is at least four times greater than the first value or "out of range" (O.D.>2); or

Two consecutive HIV p24 antigen VQA **corrected** values that are "out of range" (O.D.>2); or

Three consecutive increasing HIV p24 antigen VQA **corrected** values > 30 pg/mL, where neither consecutive value is > 4 times the previous sample, but the third value is at least four times greater than the first.

VIII. REFERENCES

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